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Characterization of an Interaction between Insulin Receptor Substrate 1 and the Insulin Receptor by Using the Two-Hybrid System

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Insulin receptor substrate 1 (IRS-1) is a major substrate of the insulin receptor and has been implicated in insulin signaling. Although IRS-1 is thought to interact with the insulin receptor, the nature of the interaction has not been defined. In this study, we used the two-hybrid assay of protein-protein interaction in the yeast *Saccharomyces cerevisiae* to study the interaction between human IRS-1 and the insulin receptor. We demonstrate that IRS-1 forms a specific complex with the cytoplasmic domain of the insulin receptor when both are expressed as hybrid proteins in yeast cells. We show that the interaction is strictly dependent upon receptor tyrosine kinase activity, since IRS-1 shows no interaction with a kinase-inactive receptor hybrid containing a mutated ATP-binding site. Furthermore, mutation of receptor tyrosine 960 to phenylalanine eliminates IRS-1 interaction in the two-hybrid assay. These data suggest that the interaction between IRS-1 and the receptor is direct and provide evidence that the juxtamembrane domain of the receptor is involved. Furthermore, we show that a 356-amino-acid region encompassed by amino acids 160 through 516 of IRS-1 is sufficient for interaction with the receptor in the two-hybrid assay. Lastly, in agreement with our findings for yeast cells, we show that the insulin receptor is unable to phosphorylate an IRS-1 protein containing a deletion of amino acids 45 to 516 when expressed in COS cells. The two-hybrid assay should provide a facile means by which to pursue a detailed understanding of this interaction.

In recent years, extraordinarily rapid advances have been made in the understanding of the molecular mechanisms by which receptor tyrosine kinases (RTKs) function. These advances are largely due to the identification of cytosolic proteins which associate with the RTK and transduce signals from the receptor to the interior of the cell. These proteins include phospholipase C-γ1, Ras–GTPase-activating protein, phosphatidylinositol 3-kinase, the SH-PTP2 phosphatase (syp), and Grb2 (11, 21, 22, 28, 29, 38). All of these proteins contain Src homology 2 (SH2) domains of approximately 100 amino acids which interact directly with specific phosphotyrosine-containing peptide domains within the RTK (24, 33). The amino acid sequence surrounding the phosphotyrosine moiety (generally only four amino acids) has been shown to be the primary determinant of the specificity of SH2 domain interaction (39). From these data has emerged a general model suggesting that hormone binding induces receptor dimerization, leading to autophosphorylation on tyrosines. This results in the recruitment of SH2-containing effector proteins to the receptor (8, 45). At this point, the effector protein is thought to be activated either by direct receptor phosphorylation or by other, perhaps conformational, changes which occur during this interaction. Alternatively, the receptor interaction might bring the effector protein to the plasma membrane, where it interacts with membrane-specific targets.

A similar yet distinct model has been proposed to explain insulin receptor signaling (20, 50). It has been known for almost a decade that stimulation of most cell types with insulin leads to the rapid tyrosine phosphorylation of a cytosolic protein with an M_r of 165,000 to 185,000 termed pp185 (52). The cDNAs encoding the rat liver and human skeletal muscle forms of pp185 have recently been cloned and renamed IRS-1, for insulin receptor substrate 1 (2, 42). IRS-1 is a cytoplasmic protein which is rapidly phosphorylated upon multiple tyrosines immediately after insulin receptor autophosphorylation. Tyrosine-phosphorylated IRS-1 interacts with a variety of SH2-containing proteins, including the p85 subunit of phosphatidylinositol 3-kinase, Grb2, and SH-PTP2 (syp) (4, 25, 38, 40), although the physiological relevance of these proteins to insulin signaling has not yet been rigorously proven. It is noteworthy that IRS-1 appears to differentiate the insulin receptor from the other classes of RTKs in which the SH2-containing proteins interact directly with the receptor. IRS-1 has been shown to be an important component of mitogenic signaling by the insulin receptor (34, 49), but the role of IRS-1 in metabolic signaling has not been clearly demonstrated.

IRS-1 appears to interact with the insulin receptor in vivo, since a small amount of IRS-1 can be coimmunoprecipitated with the insulin receptor by using antireceptor antibodies and a small amount of insulin receptor can be coimmunoprecipitated with anti-IRS-1 antibodies (4, 41). It is unclear whether this phenomenon represents a direct interaction between the receptor and IRS-1 or involves accessory proteins. Although these studies showed that insulin stimulation of the receptors was required for coimmunoprecipitation, it is unclear whether the insulin receptor must be tyrosine phosphorylated in order to interact with IRS-1. Alternatively, insulin-induced conformational changes of the insulin receptor may be responsible for the interaction. To begin to address these questions, we explored the use of the two-hybrid assay of Fields and Song (15) to detect and measure the interaction between IRS-1 and the insulin receptor. These assays allow the measurement of in vivo protein-protein interactions in the yeast *Saccharomyces cerevisiae* (10, 15, 18, 53, 54). The two-hybrid assay has been used to study the interaction of a variety of proteins, including

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the SH3-mediated interaction of Grb2 with SOS, the p21*ras* interaction with the Raf1 kinase, proteins which interact with the transcription factor serum response factor, and c-*myc* transcriptional partners (9, 12, 46, 54). Here we report the successful use of the two-hybrid assay to study in detail an interaction between human IRS-1 and the insulin receptor.

MATERIALS AND METHODS

Yeast strains and plasmids. The yeast strain EGY40 (α *trp1 ura3-52 his3 leu2*) and all yeast expression plasmids were generously provided by the laboratory of Roger Brent. The *lexAop-lacZ* reporter plasmid pSH18-34, LexA fusion plasmid pEG202, activation domain fusion plasmid pJG4-5, positive control LexA-GAL4 plasmid pSH17-4, and negative control LexA-bicoid plasmid RFHM1 have been previously described (16, 18, 54). The human skeletal muscle IRS-1 cDNA was provided by C. Ronald Kahn, and the amino acid numbering is according to Araki et al. (2).

Plasmid construction. The cDNA fusions were produced by a variety of standard methods (3). These included the use of PCR, oligonucleotide linkers, prior shuttling into one or more intermediate vectors, and filling in or removing overhangs to allow the in-frame insertion into the yeast expression vectors. Detailed cloning strategies for each clone are available upon request and will not be presented here. All junctions were sequenced by using customized primers and Sequenase 2.0 sequencing protocols (United States Biochemical Corporation, Cleveland, Ohio), as were all PCR-derived DNAs.

The amino acid sequences of the junctions are shown below. The LexA or activation domain amino acids are underlined (the initial E corresponds to the GAA codon in the *Eco*RI cloning site in both plasmids), the amino acids which encode IRS-1 or the insulin receptor are shown in boldface, and amino acids introduced by linker sequences are shown in normal type. Where appropriate, the restriction enzyme sites used to obtain these fragments are shown in parentheses. Acid blob–IRS-1 fusion sequences were as follows: 21-1242, ELIRC**K**, (*Fsp*I, IRS-1 stop); 45-516, ELIRF**E** (*Xho*I, *Bgl*II); 516-865, EF**DD**, (*Bgl*II, *Bam*HI); 865-1242, EF**DD**, (*Bam*HI, IRS-1 stop); 108-516, EF**RQ**, (*Kpn*I, *Bgl*II); 160-516, ELEFP**F**, (*Bsm*I, *Bgl*II); 203-516, EF**RA**, (*Eag*I, *Bgl*II); 258-516, ELEF**R**, (*Sfi*I, *Bgl*II); 45-456, ELIRF**E**, (*Xho*I, *Sac*II); 45-400, ELIRF**E**, (*Xho*I, *Nco*I); 45-256, ELIRF**E**, (*Xho*I, *Sfi*I); 160-456, ELEFP**F**, (*Bsm*I, *Sac*II); and 160-400, ELEFP**F**, (*Bsm*I, *Nco*I). The sequence of the LexA-insulin receptor fusion, 941-1343, was EFPGIRRHTRMHHHHHHDDDDK**R**. This Arg-941 residue from the insulin receptor refers to the first amino acid after the transmembrane domain. The insulin receptor numbering is according to Ullrich et al. (44). The receptor hybrid also contains a histidine tag and enterokinase cleavage consensus which was originally used for affinity purification of the cytoplasmic domain in unrelated experiments. The insulin receptor K1018A and Y960F mutations were made by site-directed mutagenesis using standard protocols (26). All mutants were sequenced prior to use. For the IRS-1 acid blob fusion proteins which do not contain the IRS-1 stop codon, the stop codons are provided by the yeast alcohol dehydrogenase terminator sequences downstream of the polylinker. We noted some minor discrepancies in the predicted sizes of some of the IRS-1 hybrids (see Fig. 3B, lanes 6 and 8). The reasons for this are unclear, although it seems likely that it is a reflection of the well-known anomalous behavior of IRS-1, which runs at ~180 kDa despite a predicted molecular mass of ~135 kDa.

Maintenance and transformation of yeast strains. All routine growth and maintenance of yeast strains were as described elsewhere (17). Plasmid transformation of yeast strains was either by the lithium acetate method (36) or by electroporation (5).

β-Galactosidase assays. The colony color β-galactosidase assay was performed as described previously (6) except that nylon filters were first immersed in liquid nitrogen for 5 s to permeabilize the cells. Filters were then air dried and laid onto Whatman 3MM paper which was saturated with Z buffer–5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) solution (6). Colony color assays were performed on at least five independent colonies from each hybrid. The solution β-galactosidase assays were performed as described previously (7), and the units of β-galactosidase activity were calculated by the method of Miller (31). Solution assays for the positive colonies were performed on at least two independent colonies, and the numbers within an experiment varied less than 10% between colonies. The values shown for the positive colonies represent averages of four assays (two independent colonies performed in duplicate). For the negative colonies, the numbers represent averages of two assays (single colony performed in duplicate).

Immunoblot analysis of hybrid protein expression. Overnight cultures of plasmid-containing yeast cells were grown in selective medium with 2% glucose. The following day, the cultures were pelleted, washed in sugar-free medium, diluted 1:50 into fresh selective medium with either 2% glucose or 2% galactose, and grown to an optical density at 600 nm of 1.0 to 1.3. At this point, the cells were pelleted and resuspended in sodium dodecyl sulfate (SDS) sample buffer, boiled, and subjected to SDS-polyacrylamide gel electrophoresis (PAGE) (27). The proteins were blotted to nitrocellulose (43), and the activation domain hybrid proteins were detected by using an antihemagglutinin antibody (3) and the ECL detection system (Amersham, Arlington Heights, Ill.). The insulin receptor hybrids were detected by immunoblotting with monoclonal antibody IR CT-1 (a gift from Ken Siddle). The phosphotyrosine blot was performed with antibody PY20 (Transduction Laboratories, Lexington, Ky.).

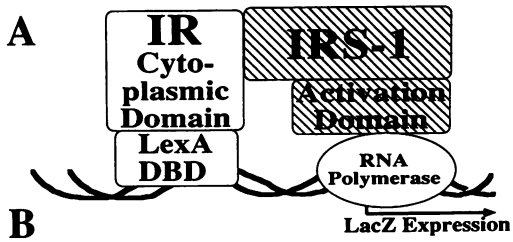
COS cell expression studies. The entire IRS-1 cDNA was transferred to the pECE mammalian expression vector (13). The Δ45-515 mutant was constructed by insertion of a linker between the *Xho*I and *Bgl*II sites in order to maintain the correct reading frame. These expression plasmids were used to transfect COS-7 cells by the DEAE-dextran procedure (3). The IRS-1 plasmids were cotransfected with an insulin receptor expression plasmid (13). On the day after transfection, the cells were split into two plates and allowed to grow for 48 h. At this point, the cells were treated for 10 min with phosphate-buffered saline supplemented with 1% bovine serum albumin with or without 100 nM insulin. After 10 min at 37°C, the medium was removed and the cells were scraped into 300 μl of SDS-PAGE sample buffer. The cell lysates were boiled for 10 min and briefly sonicated. Duplicate 7.5% gels were loaded with 30 μl of sample, and following electrophoresis, proteins were blotted to nitrocellulose and analyzed with the ECL detection system (Amersham), using either an antiphosphotyrosine antibody (PY20; Transduction Laboratories) or an antibody against the carboxy terminus of IRS-1 (Upstate Biotechnology Inc., Lake Placid, N.Y.).

RESULTS

The two-hybrid assay demonstrates specific interaction of IRS-1 with the insulin receptor. To test the utility of the two-hybrid assay in the measurement of an interaction between the insulin receptor and IRS-1, we used the interaction trap system (18, 54), a modified version of the two-hybrid

system first described by Fields and coworkers (10, 15). These assays are based upon the finding that transcription factors contain separable functional modules which direct either DNA binding or transcriptional activation. The cDNA of the protein of interest is used to make a fusion with a DNA-binding domain from a known DNA-binding protein such as LexA. These DNA-binding hybrids cannot activate transcription since they do not contain a transcriptional activation domain. A second protein suspected to interact with the first protein is fused to a known transcriptional activation domain such as an acid blob domain. This hybrid protein can activate transcription but cannot by itself interact with DNA. Both of these hybrid proteins are expressed in yeast cells. The DNA-binding bait hybrid interacts with the DNA at specific sequences located upstream of a marker gene such as *lacZ*, whose protein product, β-galactosidase, can be easily assayed. If the second activation domain hybrid associates with the bait hybrid within the nucleus, the activation domain is held in the vicinity of the promoter and transcription of the marker gene is activated.

To construct the bait hybrid protein, we fused the cytoplasmic domain of the insulin receptor to the DNA-binding region of LexA. This hybrid contains the LexA DNA-binding domain at the amino terminus followed by the entire cytoplasmic domain of the insulin receptor (amino acids 941 to 1343 according to Ullrich et al. [44]). To produce the second hybrid, we fused amino acids 21 to 1242 of human IRS-1 (all but the first 20 amino acids) to the acidic activation domain. At its amino terminus, this IRS-1 hybrid protein contains the simian virus 40 large T antigen nuclear localization signal, the influenza virus HA1 epitope tag, and the B42 acidic transcriptional activation domain, which is also referred to as an acid blob domain (18, 54), followed by the IRS-1 sequences. A schematic of these hybrid proteins is shown in Fig. 1A. Plasmids expressing these hybrid proteins were introduced into yeast strain EGY40 along with the *lexAop-lacZ* reporter plasmid, and transformants were identified by growth on the proper selective media. These colonies were first analyzed by the colony filter assay for β-galactosidase activity (6) after replica plating onto selective plates containing either glucose or galactose (in order to induce the galactose-specific expression of the IRS-1 fusions) (18). Since the GAL promoter is repressed by glucose and induced by galactose, β-galactosidase activity should be observed only when the yeast cells are grown in the presence of galactose. As summarized in Fig. 1B, no β-galactosidase activity was observed when the insulin receptor hybrid was expressed independently of the IRS-1 hybrid, regardless of the carbon source. Conversely, when the IRS-1 hybrid was coexpressed with the insulin receptor bait, blue colonies were observed, but only when cells were grown on galactose-containing plates. To ensure that the IRS-1 hybrid was not interacting with the LexA portion of the bait, it was coexpressed with a second bait hybrid protein which contains the identical LexA domain fused to a portion of the *Drosophila* bicoid protein (54). No β-galactosidase activity was observed with this combination, demonstrating that the IRS-1 hybrid does not interact with the LexA portion of the bait hybrid. In addition, this result shows that the IRS-1 hybrid cannot induce β-galactosidase activity by itself. To better quantitate β-galactosidase activity, solution assays were performed with cell lysates from cells grown in either glucose or galactose. As shown in Fig. 1B, these results confirmed those obtained from the colony color filter assay. Coexpression of the IRS-1 and insulin receptor hybrids resulted in an approximately 100-fold increase in activity when cells were grown in galactose. Both assays of β-galactosidase activity showed clear and reproducible differences between glucose- and galactose-grown cells. To



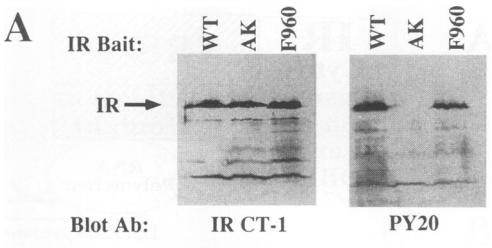
Plasmids			Colony Color		β-Gal Activity	
LacZ Reporter	LexA Hybrid	Activation Hybrid	GLU	GAL	GLU	GAL
+	IR	-	-	-	2	2
+	IR	IRS-1	-	++++	2	209
+	Bicoid	IRS-1	-	-	2	2
+	GAL4	-	++++	++++	727	532

FIG. 1. IRS-1 interacts specifically with the insulin receptor in the two-hybrid assay. (A) Schematic representation of the hybrid proteins. The entire cytoplasmic domain of the human insulin receptor (IR) was fused to the LexA DNA-binding domain (DBD). The cDNA encoding amino acids 21 to 1242 of IRS-1 was fused to the acid blob activation domain. Interaction of these hybrid proteins would be expected to drive the expression of the *lexAop-lacZ* reporter gene. (B) The insulin receptor hybrid and the *lexAop-lacZ* reporter were introduced into yeast cells in the absence or presence of the IRS-1 hybrid and transformants were isolated on selective plates. Transformants were assayed for β-galactosidase (β-Gal) activity by either the colony color or the solution assay as described in Materials and Methods. As a negative control, the IRS-1 21-1242 hybrid was coexpressed with the LexA-bicoid hybrid. As a positive control, the GAL4 activation domain-LexA hybrid was coexpressed with the *lexAop-lacZ* reporter. The colony color assay showed either white (−) or dark blue (++++) colonies.

compare the activity that we observed to that of a known activator of the *lexAop-lacZ* reporter, we expressed a LexA-GAL4 fusion protein which is known to induce high-level activity. The hybrid LexA-GAL4 protein is constitutively expressed and gives high activity when grown on glucose or galactose (18). The activity generated by the insulin receptor interaction with IRS-1 was consistently 30 to 40% of the level observed with the positive control. Considering that the LexA-GAL4 hybrid is monomeric and that the GAL4 activation domain is generally considered to be a stronger transcriptional activator than the acid blob domain in our constructs (18), it appears that the β-galactosidase activity generated by the insulin receptor interaction with IRS-1 is quite high. We conclude that IRS-1 forms a specific interaction with the insulin receptor in the yeast nucleus which can be easily measured in the two-hybrid system, using either the colony color or the solution assay to measure β-galactosidase activity.

Insulin receptor kinase activity is essential for IRS-1 interaction. We next sought to determine whether the state of tyrosine phosphorylation of the insulin receptor was important for the interaction with IRS-1. In this regard, mutation of the critical lysine (K-1018) within the ATP-binding motif of the insulin receptor has been shown to eliminate receptor activity and IRS-1 phosphorylation (20). It is unclear whether this receptor mutation has any effect upon the interaction with IRS-1 or whether this mutant receptor simply cannot phosphorylate IRS-1. To test these possibilities in the two-hybrid system, we mutated Lys-1018 to Ala within the insulin receptor-LexA bait hybrid by site-directed mutagenesis. To be sure that the receptor ATP-binding mutant (termed the AK mu-

A



B

Plasmids		Colony Color		β-Gal Activity	
LexA Hybrid	Activation Hybrid	GLU	GAL	GLU	GAL
IRWT	IRS-1	-	++++	2	112
IRAK	IRS-1	-	-	2	4
IRF960	IRS-1	-	-	2	3

FIG. 2. Mutation of either the ATP-binding site or Tyr-960 within the juxtamembrane domain of the insulin receptor eliminates IRS-1 interaction in the two-hybrid system. (A) Immunoblot analysis of expression and tyrosine phosphorylation of the wild-type (WT), ATP-binding mutant K1018A (AK), or the Y960F mutant (F960) receptor hybrids was performed as described in Materials and Methods. Duplicate lanes were blotted and analyzed either with the insulin receptor (IR)-specific antibody (Ab) IR CT-1 or the antiphosphotyrosine antibody PY20. The receptor hybrid location is shown by the arrow. (B) The IRS-1 21-1242 hybrid was introduced into yeast cells in the presence of either the wild-type insulin receptor (IRWT)-LexA hybrid or an insulin receptor-LexA hybrid which had been mutated within the ATP-binding domain (IRAK) or within the juxtamembrane domain (IRF960). Transformants were assayed for β-galactosidase (β-Gal) activity by either the colony color or the solution assay as described in Materials and Methods. The colony color assay showed either white (−) or dark blue (++++) colonies.

tant) was being properly expressed and was kinase inactive, we analyzed its expression and phosphorylation state by immunoblot analysis using either an antireceptor or antiphosphotyrosine antibody. As shown in Fig. 2A, the AK mutant showed levels of expression similar to those of the wild-type receptor by immunoblotting with the antireceptor antibody IR-CT1. Despite similar levels of expression, the AK mutant showed no tyrosine phosphorylation compared with the wild-type receptor, as demonstrated by immunoblotting a duplicate blot with the antiphosphotyrosine antibody PY20. We next tested whether the 21-1242 IRS-1 hybrid interacted with this AK mutant receptor in the two-hybrid assay. As shown in Fig. 2B, the AK receptor mutant showed no significant interaction with the IRS-1 hybrid. Importantly, these data also demonstrate that the wild-type receptor cytoplasmic domain retains the ability to undergo autophosphorylation when expressed as a hybrid in yeast nuclei and show directly, for the first time, that receptor kinase activity is essential for IRS-1 interaction.

Tyrosine 960 within the receptor juxtamembrane domain is essential for IRS-1 interaction. Mutations within the receptor juxtamembrane domain have been shown to greatly decrease IRS-1 phosphorylation (51). Although this region of the receptor has been postulated to be the site of IRS-1 interaction, this has not been shown directly. To address this question, we mutated Tyr-960 to Phe in the receptor hybrid. As shown in Fig. 2A, this receptor mutant showed levels of expression similar to those of the wild-type receptor. The Y960F mutant receptor retained the ability to autophosphorylate, although with decreased total phosphorylation compared with the wild-type receptor. These results would be predicted from previous studies done with higher eukaryotic cells in which this same mutation was introduced into the intact insulin receptor (51). We next tested the ability of the Y960F receptor hybrid to interact with the IRS-1 21-2142 hybrid in the two-hybrid assay. As shown in Fig. 2B, this mutant receptor showed no interaction with IRS-1. Our data provide direct support for the widely held theory that the juxtamembrane domain, and specifically Tyr-960, forms a part of the IRS-1 binding site and, together with the ATP-binding receptor mutant data, suggest that receptor autophosphorylation of Tyr-960 is essential for this interaction.

Identification of a region of IRS-1 which is important for interaction with the insulin receptor. Having demonstrated the utility of the two-hybrid assay in accurately reproducing an interaction between IRS-1 and the insulin receptor, we were interested in delineating the region of IRS-1 which interacts with the insulin receptor. We therefore expressed a series of relatively large regions of IRS-1 as activation hybrids and examined whether they interacted with the insulin receptor in the two-hybrid assay. These constructs and their locations relative to full length IRS-1 are shown schematically in Fig. 3A. We found that a region bordered by amino acids 45 and 516 was sufficient for receptor interaction and that sequences C terminal of amino acid 516 showed little or no interaction with the insulin receptor. As a further test, we expressed a 516-1242 hybrid as well as two hybrids which contained deletions within the 45-516 region. All of these constructs showed little activity. To examine whether the low levels of activity of the 516-865, 865-1242, 516-1242, and the two deletion hybrids were not simply due to decreased expression, we examined their galactose-inducible expression by immunoblotting. As shown in Fig. 3B, when cell lysates were immunoblotted with the hemagglutinin antibody, all seven hybrids were found to be expressed in a strictly galactose inducible manner at roughly similar levels. We conclude that the hybrid protein containing amino acids 45 to 516 is sufficient for receptor interaction and that other domains of IRS-1 show much less interaction with the receptor despite high-level protein expression. Our finding that the three hybrids which contain the 516-1242 region show slight activity in the colony filter assay suggests that this region of IRS-1 may also contain a weaker domain of interaction.

To further delineate the region of IRS-1 which interacts with the insulin receptor, we assayed nine constructs within the 45-516 region for receptor interaction. Amino-terminal deletions suggest that amino acids between 160 and 203 are critical for interaction. Specifically, as shown in Fig. 4A, the 108-516 and 160-516 hybrids showed dark blue colonies and high-level β-galactosidase activity which was generally comparable to that of the parental 45-516 hybrid. The 45-516 hybrid gave consistently higher activities than did the 108-516 and 160-516 hybrids, yet it is unclear whether this is significant. It is therefore possible that the 45-108 region contributes to the interaction. Further deletion of either amino acids 160 to 203 or 160 to 258 eliminated activity, suggesting that an essential component of interaction is located between amino acids 160 and 203. The C-terminal boundary of interaction appears to be somewhat more complex, as demonstrated by C-terminal deletions. One element which is required for high-level activity appears to be located between amino acids 456 and 516, since deletion of amino acids 457 through 516 or 401 through 516 showed much reduced (~90 to 95%) though significant activity. By itself, this element is totally inactive and appears to require the 160-203 essential region, as shown by the lack of activity of the 203-516 and 258-516 hybrids. The 45-256 fragment retains only very slight activity in both the colony

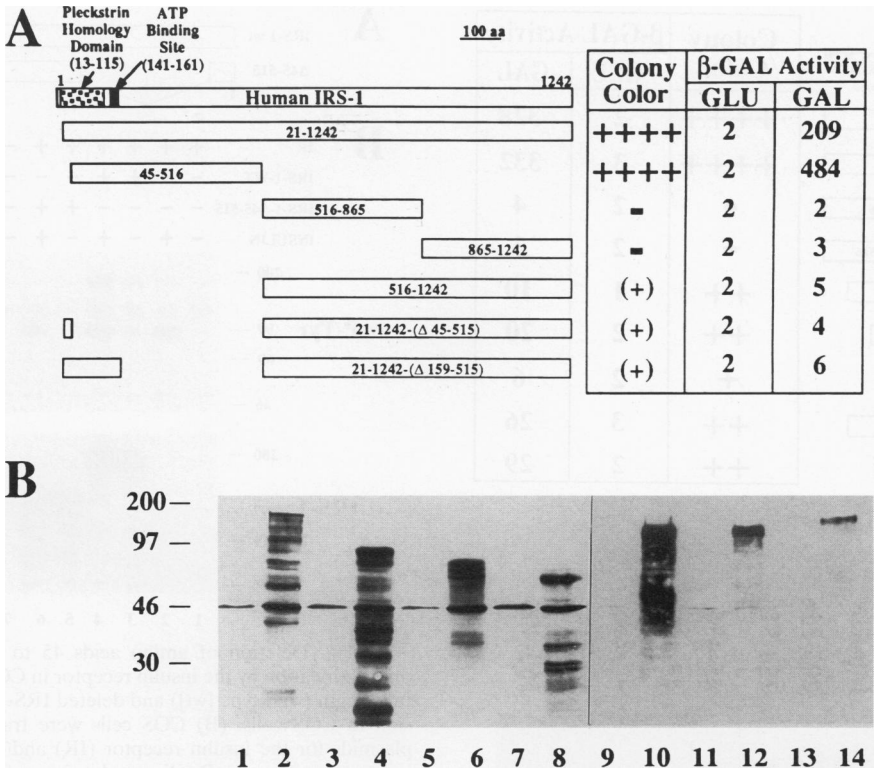


FIG. 3. The amino-terminal region of IRS-1 interacts with the insulin receptor. (A) Schematic of four IRS-1 activation domain hybrids used in the two-hybrid assay. The colony color assay showed either white (−), very light blue [(+)], or dark blue (++++) colonies when cells were grown on galactose-containing plates. When cells were grown on glucose-containing plates, all colonies were white (data not shown). The β-galactosidase (β-GAL) activity was also quantitated by using the solution assay as described in Materials and Methods. The locations of the ATP-binding and PH domains are shown at the top. This experiment was performed simultaneously with that shown in Fig. 1B, and the data for the 21-1242 hybrid are therefore identical. aa, amino acids. (B) Immunoblot analysis of expression of the IRS-1 hybrid proteins. Expression of the IRS-1 hybrids was analyzed by immunoblotting with the hemagglutinin epitope tag antibody as described in Materials and Methods. Lanes: 1 and 2, hybrid 21-1242; 3 and 4, hybrid 45-516; 5 and 6, hybrid 516-865; 7 and 8, hybrid 865-1242; 9 and 10, hybrid 516-1242; 11 and 12, 21-1242-(Δ45-515); 13 and 14, 21-1242-(Δ159-515). The cultures were grown with either glucose (odd-numbered lanes) or galactose (even-numbered lanes). The extracts used for immunoblotting were derived from the same yeast cultures that were used in the solution β-galactosidase assays. Sizes are indicated in kilodaltons.

color assay and the solution assay, suggesting either that the critical element located between 160 and 203 retains a slight activity in the absence of the C-terminal elements or that amino acids 45 to 108 are involved. The smallest peptide within this region which retained significant activity included amino acids 160 through 400. These experiments suggest that this insulin receptor-binding element of IRS-1 is composed of multiple interdependent components which mediate the interaction in a combinatorial manner.

To rule out the possibility that the differences in activity were due to differences in levels of protein expression, we examined the expression of the IRS-1 hybrids by immunoblotting as described above. As shown in Fig. 4B, all of the IRS-1 activation domain fusions were expressed. The proteins were generally of the sizes predicted (Fig. 4B), and all showed galactose inducibility (data not shown). It appeared that all hybrid proteins were being properly expressed, and expression levels appeared to be generally similar. These experiments demonstrate that the fragments which showed no activity in the two-hybrid assay (203-516 and 258-516; Fig. 4B, lanes 3 and 4) are expressed at high levels. It is therefore likely that the negative results in the two-hybrid assay are due to the inability of these hybrids to interact with the insulin receptor. All of the hybrids showed numerous bands when immunoblotted, presumably as a result of proteolytic degradation. The proteolysis is perhaps not surprising, since the yeast cells are grown for relatively long times (18 to 24 h) prior to extract preparation, and may therefore simply represent normal protein turnover. This proteolysis does not appear to affect the interaction, since there is no correlation between degree of proteolysis and lack of activity.

Deletion of amino acids 45 to 516 eliminates IRS-1 phosphorylation by the insulin receptor in COS cells. To test further our assertion that amino-terminal sequences of IRS-1 mediate the interaction with the insulin receptor, we used a COS cell transient expression system. We reasoned that if this region of IRS-1 was important for receptor interaction, a mutant IRS-1 molecule containing a deletion of this region should be a relatively poor insulin receptor substrate. We therefore cotransfected COS cells with vectors designed to coexpress the insulin receptor with either the full-length IRS-1 or a deletion mutant IRS-1 from which amino acids 45 to 515 had been deleted (Fig. 5A). We then examined whether these IRS-1 proteins were phosphorylated on tyrosines in response to insulin treatment of the cells. As shown in Fig. 5B, the insulin receptor transfectants showed a high level of insulin-dependent receptor autophosphorylation by antiphosphotyrosine immunoblotting (top, lanes 2, 4, and 6) compared with

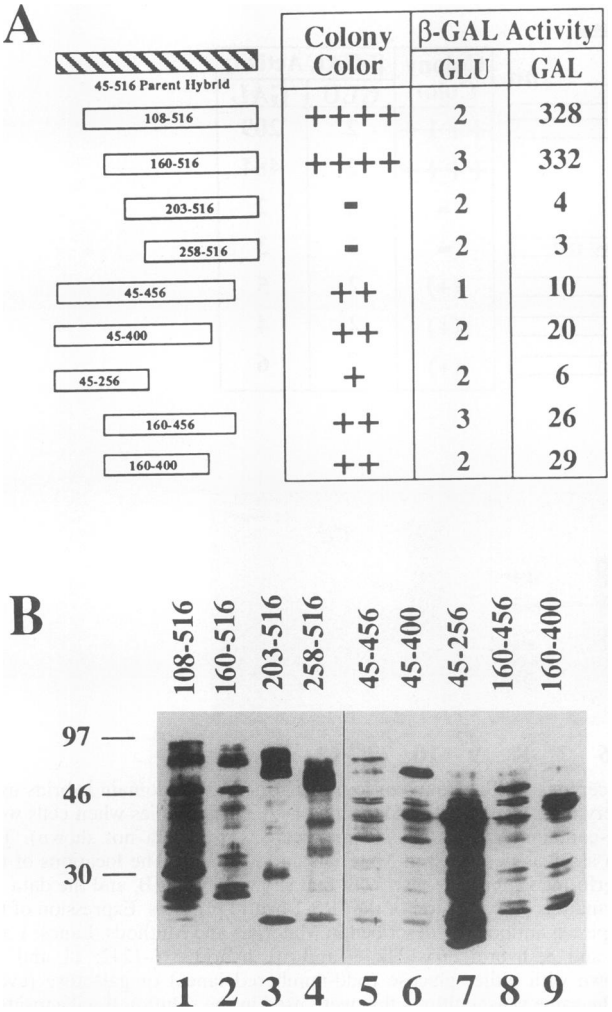


FIG. 4. Delineation of the domain of IRS-1 which interacts with the insulin receptor. (A) Schematic of nine IRS-1 activation domain hybrids used in the two-hybrid assay. The structure of the parent 45-516 hybrid is shown at the top for comparison. The colony color assay showed either white (−), dark blue (++++), medium blue (++), or light blue (+) colonies when cells were grown on galactose plates. When cells were grown on glucose-containing plates, all colonies were white (data not shown). The β-galactosidase (β-GAL) activity was quantitated by using the solution assay as described in Materials and Methods. (B) Immunoblot analysis of expression of the IRS-1 activation domain hybrid proteins was performed as described in the legend to Fig. 2B and in Materials and Methods except that only galactose-induced extracts are shown. The activation domain hybrids assayed in lanes 1 to 9 are shown above the gel. The extracts used for immunoblotting were derived from the same yeast cultures that were used in the solution β-galactosidase assays. Sizes are indicated in kilodaltons.

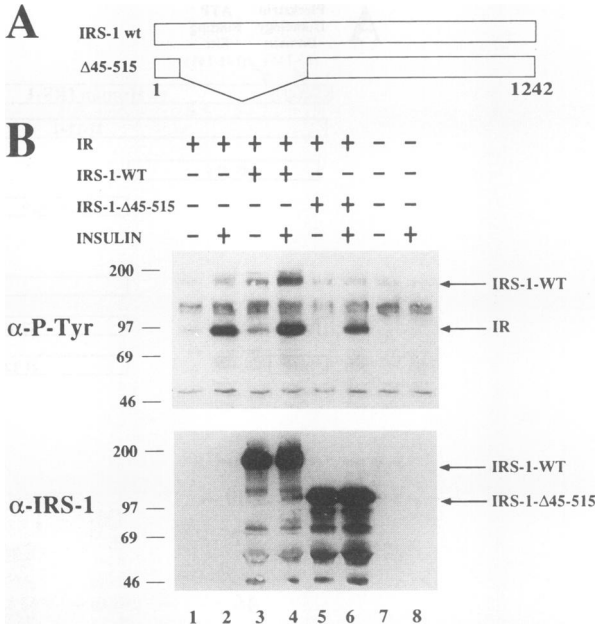


FIG. 5. Deletion of amino acids 45 to 515 of IRS-1 eliminates phosphorylation by the insulin receptor in COS cells. (A) Schematic of full-length (wild-type [wt]) and deleted IRS-1 cDNAs used for expression in COS cells. (B) COS cells were transfected with expression plasmids for the insulin receptor (IR) and/or the IRS-1 plasmids as indicated at the top. Duplicate plates were treated with 0 or 100 nM insulin as shown, and lysates were analyzed by immunoblotting with either an antiphosphotyrosine (α-P-Tyr) or anti-IRS-1 (α-IRS-1) antibody as described in Materials and Methods. Lanes 7 and 8 represent mock-transfected cells. Locations of the insulin receptor (IR) and wild-type IRS-1 (IRS-1-WT) proteins are shown by the arrows. Sizes are indicated in kilodaltons.

mock-transfected cells (lane 8). When coexpressed with the insulin receptor, the full-length IRS-1 protein was phosphorylated upon tyrosines at a level significantly above that of endogenous IRS-1 (compare lane 4 with lane 2). Conversely, when the IRS-1 Δ45-516 deletion was coexpressed, no tyrosine phosphorylation of a band corresponding to the mutant IRS-1 protein was observed (lane 6) despite normal insulin receptor autophosphorylation. To be sure that the two IRS-1 proteins were being expressed at similar levels, a duplicate blot was analyzed with an anti-IRS-1 antibody. As shown in Fig. 5B, both IRS-1 expression constructs showed high-level expression of proteins of the predicted molecular weight. The IRS-1 deletion protein ran considerably more slowly than the insulin receptor, and the lack of tyrosine phosphorylation is therefore not a result of comigration with the receptor. Longer exposures do not reveal any phosphorylation of a band corresponding to the IRS-1 deletion mutant (data not shown). These results suggest that the amino-terminal region of IRS-1 is required for efficient interaction with the insulin receptor in higher eukaryotic cells and is not simply an artifact of the two-hybrid assay in yeast cells.

DISCUSSION

Since IRS-1 is likely to be an important mediator of at least some of the effects of the insulin receptor, the regulation of the interaction between the receptor and IRS-1 is of primary importance to insulin signaling. In this paper, we report the successful use of the two-hybrid assay in *S. cerevisiae* to detect and begin to characterize the interaction between IRS-1 and the insulin receptor. Since this assay has not been used previously to study interactions of proteins with active tyrosine kinases, it was critical to first demonstrate that the cytoplasmic domain of the insulin receptor could be expressed in yeast cells in a properly folded, enzymatically active form. Our demonstration that the wild-type and Y960F receptor hybrids retain the ability to autophosphorylate on tyrosines (Fig. 2) shows that the hybrid proteins are properly processed and are enzymatically active in yeast cells. We hypothesize that the

autophosphorylation may be due in part to the fact that the LexA portion of the hybrid binds to the DNA as a dimer, and thus the receptors have the opportunity to autophosphorylate in an intermolecular manner. Our demonstration that mutations within the ATP-binding and juxtamembrane domains of the insulin receptor which are known to interfere with IRS-1 phosphorylation in vivo also show no interaction with IRS-1 in our yeast system argues strongly that the interaction which we are able to measure in the yeast system is similar to that which occurs in higher eukaryotic cells. In further support of this argument, we show (Fig. 5) that deletion of the interacting domain of IRS-1 identified in *S. cerevisiae* (amino acids 45 to 515) eliminates IRS-1 phosphorylation by the insulin receptor in COS cells. Since the majority of the tyrosines which undergo insulin receptor-mediated phosphorylation are located between amino acids 516 and 1242 (40), it is unlikely that the lack of phosphorylation of this mutant is due the deletion of tyrosines. We conclude from these data that the two-hybrid system accurately reconstitutes at least one interaction between the receptor and IRS-1 and is thus a valid model for further study.

It has been shown that a small amount of IRS-1 can be coimmunoprecipitated from cell lysates by using antireceptor antibodies and likewise that a small amount of insulin receptor can be coimmunoprecipitated with anti-IRS-1 antibodies under mild conditions (4, 41). It cannot be determined from this type of study whether the interaction is direct or is mediated by accessory proteins. Our data suggest that at least one interaction between IRS-1 and the insulin receptor is direct, although we cannot entirely rule out the possibility that this interaction is mediated by endogenous yeast proteins. This seems unlikely, since yeasts have not been reported to contain signaling systems such as the insulin receptor or IRS-1 and thus would not be expected to express accessory proteins which might be involved in this interaction. In addition, even if such accessory proteins do exist in *S. cerevisiae*, it is unlikely that they would be localized to the nucleus. We speculate that the interaction between the insulin receptor and IRS-1 is not as stable as that which occurs between RTKs and SH2 domain-containing proteins. In this regard, using insulin soluble cytoplasmic domain receptor as probes, we were unable to demonstrate a clear interaction with blotted IRS-1, whereas we were able to easily demonstrate interaction of the insulin receptor probes with blotted SH2 domain-containing p85 proteins (data not shown). Perhaps the interaction with IRS-1 has a lower affinity or a higher off-rate compared with SH2 interactions, or this interaction may be sensitive to the detergents commonly used in immunoprecipitations and filter blot assays. In this regard, the two-hybrid assay may be more sensitive than other assays, since theoretically it is not necessary that an interaction be maintained for an extended period of time because even transient interactions should allow transcriptional initiation of the reporter gene.

It has been proposed that the juxtamembrane domain of the insulin receptor is the site of IRS-1 interaction, since mutations within this domain eliminate phosphorylation of IRS-1 without significantly affecting autophosphorylation of the remainder of the receptor (19, 35, 51). Specifically, deletions within the juxtamembrane domain or mutation of Tyr-960 to Phe eliminated IRS-1 phosphorylation (19, 51). These results suggest that Tyr-960, which is phosphorylated in vivo (14), may be involved in IRS-1 interaction. Our data provide support for the theory that the juxtamembrane domain is involved in a direct interaction with IRS-1 and furthermore that phosphorylated Tyr-960 is critical for this interaction. Interestingly, these findings are consistent with an SH2 interaction, yet IRS-1 does not contain a recognizable SH2 domain. It will be interesting to determine if IRS-1 contains a distinct SH2-like motif which can also recognize phosphotyrosine-containing peptides. In this regard, it is interesting that the critical amino acids (in addition to the phosphotyrosine) which determine the specificity of SH2 interaction are thought to be located within the first three or four amino acids on the C-terminal side of the tyrosine (24, 33). Conversely, the NPXY motif contains conserved residues on the N-terminal side of the tyrosine. Thus, it is possible that IRS-1 contains a motif which recognizes phosphotyrosine-containing peptides in a manner reminiscent of SH2 domains yet recognizes slightly different sequences. In addition to our contention that IRS-1 may interact directly with the juxtamembrane domain, it is equally possible that phosphorylation of Tyr-960 alters the conformation of the insulin receptor in such a way to allow IRS-1 to interact.

It has recently been shown that the interleukin 4 (IL-4) receptor appears to mediate at least some of its effects via the tyrosine phosphorylation of an IRS-1-like molecule termed 4PS (48). These two proteins appear to share similar functions, since expression of IRS-1 has been shown to confer IL-4 responsiveness upon a normally nonresponsive cell line, 32D, which does not express 4PS (47). The 4PS protein appears to be a related but distinct gene product, since the majority of anti-IRS-1 antibodies do not recognize 4PS. The signaling component of the IL-4 receptor contains an NPXY sequence motif surrounding Tyr-497, which is very similar to the motif found in the insulin receptor, and it has been demonstrated to be critical for 4PS/IRS-1 phosphorylation in response to IL-4 (23). Furthermore, a glutathione *S*-transferase fusion protein containing this NPXY domain from the IL-4 receptor retains the ability to coprecipitate a small amount of 4PS/IRS-1 from cell lysates. It is not clear from these studies whether phosphorylation of Tyr-497 was required for this effect. Our data suggest that tyrosine phosphorylation of Tyr-497 of the IL-4 receptor via an intermediate Src-like kinase may provide a binding site for 4PS which is analogous to that which we have demonstrated for the insulin receptor. The demonstration that multiple IRS-1-like proteins exist and that they appear to interact with unrelated receptor types in a similar manner suggests that this type of protein-protein interaction may be a general phenomenon.

We show that the interaction with the insulin receptor can be mediated by a domain of IRS-1 located between amino acids 45 and 516. Deletion analysis of this region suggests that between amino acids 160 and 516 there are two important elements of interaction, located between amino acids 160 and 203 and between amino acids 456 and 516. The 160-203 domain appears to be absolutely critical for the interaction. The 456-516 domain appears to account for >90% of the activity observed, but its deletion does not entirely eliminate activity. Both domains appear to be necessary for high-level activity. Although the deletion analysis (Fig. 4) seems to clearly delineate the boundaries of the domain of interaction, we cannot rule out the possibility that structural differences between the hybrids account for some of the differences in activity. For example, certain deletions which contain a good insulin receptor interaction domain may be misfolded and therefore give artificially low activities. In this regard, our conclusions regarding the delineation of the boundaries and subdomains of interaction are strengthened by the fact that in most cases there are at least two hybrids which show similar activities. For example, the finding that the 45-456, 45-400, 160-456, and 160-400 hybrids show similar low levels of activity, compared with the four hybrids (21-1242, 45-516, 108-516, and 160-516), which show high levels of activity, serves to highlight

the importance of the 456-516 domain. Likewise, the finding that the 203-516 and 258-516 hybrids show good protein expression yet are completely negative in the two-hybrid assay suggests that the lack of β-galactosidase activity is due to a lack of interaction with the insulin receptor.

The pleckstrin homology (PH) domain located between amino acids 13 and 115 is a putative domain of protein-protein interaction of unknown function which has recently been identified in a variety of proteins involved in signaling (30, 32, 37). Our data suggest that this domain is not necessary for interaction with the insulin receptor in the two-hybrid assay. In addition to the data shown in Fig. 3 and 5, we have observed no activity of an IRS-1 (1-121) hybrid containing the entire PH domain (data not shown), suggesting that the PH domain is not sufficient for activity in our assay. Nevertheless, since this region may be somehow constrained by its direct fusion to the activation domain sequences, it is possible that this domain is involved in receptor interaction. Alternatively, the PH domain may be involved in interactions with other cellular proteins which help to mediate receptor interaction or signaling.

We have begun to examine the state of tyrosine phosphorylation of the IRS-1 hybrid proteins expressed in yeast cells. In preliminary experiments (not shown), we have observed tyrosine phosphorylation of many of the IRS-1 hybrids in yeast cells, irrespective of whether the hybrids showed activity in the two-hybrid assay. This is apparently due to the overexpression of a constitutively active receptor kinase combined with long-term high-level expression of the IRS-1 hybrids. During the ~24 h of galactose induction of the IRS-1 hybrids, the kinase is able to phosphorylate these hybrids. This phosphorylation of IRS-1 hybrids is not surprising, since the many YXXM motifs within IRS-1 are excellent substrates of the insulin receptor. Phosphorylation of the IRS-1 hybrids probably occurs throughout the nucleus and to some extent in the cytoplasm, since the majority of the receptor hybrids would not be bound to the few operators on the reporter plasmids. It is therefore important to note that tyrosine phosphorylation of a substrate is not necessarily a reflection of a stable interaction with the receptor, especially in artificial systems such as this in which substrates and receptors are overexpressed. We hypothesize that in addition to the IRS-1 sequence-dependent interaction which we are able to measure with the two-hybrid assay, interactions also occur with the constitutively activated kinase which are sequence independent and result in phosphorylation of the IRS-1 hybrids. These interactions may be analogous to the phosphorylation of short synthetic tyrosine-containing peptides such as poly(Glu-Tyr)$_{4:1}$ by the insulin receptor. These synthetic peptides are excellent receptor substrates in vitro yet almost certainly do not contain specific motifs which would direct interaction with a particular RTK. It is notable that these sequence-independent interactions do not appear to give any significant activity in the two-hybrid assay, and thus the sequence-dependent interactions such as that involving IRS-1 amino acids 45 to 516 may represent a more stable interaction. In this regard, it is perhaps surprising that we did not observe phosphorylation of the IRS-1 Δ45-515 hybrid in COS cells (Fig. 5). We hypothesize that during the limited time in which the insulin receptor is activated in these experiments (10 min), the deletion mutant is unable to efficiently interact with the receptor whereas the full-length IRS-1 interacts either more efficiently or more stably, thus allowing phosphorylation to occur. Further experiments will be required to fully examine these issues.

It is unclear whether alterations of the interaction between IRS-1 and the insulin receptor may be involved in disease states such as diabetes and insulin resistance. Interestingly, one

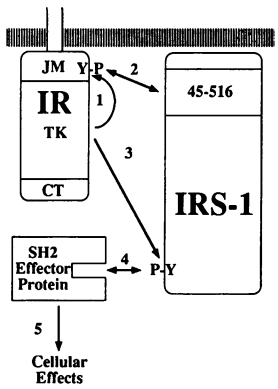


FIG. 6. Model of insulin receptor signaling via IRS-1. In this model, insulin stimulates receptor (IR) autophosphorylation, which phosphorylates Tyr-960 within the juxtamembrane domain. Phosphorylation of this tyrosine results in the direct interaction with the domain of IRS-1 encompassed by amino acids 45 to 516. This interaction may allow the proper presentation of IRS-1 to the receptor kinase domain, resulting in phosphorylation of IRS-1 on multiple tyrosines. These phosphotyrosines within IRS-1 would then interact with SH2-containing effector proteins, resulting in their activation.

mutation of IRS-1 within the region of interaction that we have delineated which appears to have a greater prevalence in type II diabetics has been identified. Specifically, an amino acid polymorphism (Ala-512 to Pro) was found in IRS-1; 7% of patients with non-insulin-dependent diabetes mellitus were found to be heterozygous for this mutation, compared with 2.6% of normoglycemic controls (1). This mutation falls within the carboxyl end of the region, which we propose to be important for interaction with the insulin receptor. Whether this mutation has any functional consequences to insulin signaling is unclear.

Our data suggest a model for insulin receptor signaling via IRS-1, which is shown schematically in Fig. 6. In this model, the insulin-activated receptor autophosphorylates Tyr-960 as part of the autophosphorylation cascade in an intermolecular manner. This phosphorylation would then direct the interaction of the receptor with the amino terminus of IRS-1. This interaction might then allow the proper presentation of IRS-1 to the already activated insulin receptor kinase domain, resulting in phosphorylation of IRS-1 on a number of tyrosines. These phosphotyrosines on IRS-1 could then interact with SH2-containing effector proteins such as phosphatidylinositol 3-kinase or Grb2 and presumably continue the signaling processes. It is notable that the tyrosines of IRS-1 which appear to be phosphorylated by the insulin receptor and subsequently interact with SH2-containing molecules such as p85 (Tyr-612 and Ty-941), SH-PTP2 (syp) (Tyr-1169), and Grb2 (Tyr-896) appear to be primarily located C-terminal of the region that we have localized (38, 40).

The precise molecular nature of the IRS-1–insulin receptor interaction remains undefined, yet we can conclude that the ATP-binding motif located at positions 141 to 161 is not required for receptor interaction, since its deletion had no effect on activity. Cursory examination of the protein sequence within the 45-516 domain did not reveal any significant homology to any known proteins in the protein databases. We conclude that this domain mediates a previously uncharacterized type of protein-protein interaction. The two-hybrid assay should provide a straightforward means to further characterize this interaction, particularly by random-point mutagenesis,

since mutants which do not interact should be easily discernible in the colony color assay.

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